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(71) Applicant *(for all designated States except US)*: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NORMAN, Barrie, Edmund,** [GB/DK]; Magnoliavej 5B, DK-3460 Birkeroed (DK). **BISGAARD-FRANTZEN, Henrik** [DK/DK]; Elmevænget 8B, DK-BAGSVÆRD 2880 (DK). **HASEMANN, Anders** [DK/DK]; Korsgade 20, 1.tv., DK-2200 Copenhagen N. (DK). **FESTERSEN, Rikke, Monica,** [DK/DK]; Nansensgade 75 A, 2 th., DK-1366 Copenhagen N (DK).

(74) Common Representative: **NOVOZYMES A/S**; Patents, Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

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(54) Title: MASHING PROCESS

(57) Abstract: The present invention relates to a process and to a composition for use in the production of a wort suitable for fermenting to a "low carb" or super attenuated beer.

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MASHING PROCESS

FIELD OF THE INVENTION

The present invention relates, inter alia, to a process for the production of an alcoholic beverage, such as beer, having a low carbohydrate content, and to a composition useful in such a process.

BACKGROUND OF THE INVENTION

The use of enzymes in brewing is common. Application of enzymes to reduce the carbohydrate content of a beer is described in US 4,666,718. However, there is a need for improved processes for production of a beer having a low carbohydrate content, i.e. a low carbohydrate beer.

SUMMARY OF THE INVENTION

The invention provides in a first aspect a process for production of a beer having a low content of carbohydrates which comprises; a) preparing a mash in the presence of enzyme activities, b) filtering the mash to obtain a wort, and, c) fermenting said wort to obtain a beer, wherein the enzyme activities comprise; an alpha-amylase, a glucoamylase and an isoamylase.

In a second aspect the invention provides a composition comprising an alpha-amylase, a glucoamylase and an isoamylase.

DETAILED DESCRIPTION OF THE INVENTION

Brewing processes are well-known in the art, and generally involve the steps of malting, mashing, and fermentation. In the traditional brewing process the malting serves the purpose of converting insoluble starch to soluble starch, reducing complex proteins, generating colour and flavour compounds, generating nutrients for yeast development, and the development of enzymes. The three main steps of the malting process are steeping, germination, and kilning.

Steeping includes mixing the barley kernels with water to raise the moisture level and activate the metabolic processes of the dormant kernel. In the next step, the wet barley is germinated by maintaining it at a suitable temperature and humidity level until adequate modification, e.g. such as degradation of starch and activation of enzymes, has been achieved. The final step is to dry the green malt in the kiln.

Mashing is the process of converting starch from the milled barley malt and solid adjuncts into fermentable and unfermentable sugars to produce wort of the desired composition. Traditional mashing involves mixing milled barley malt and adjuncts with water at a set temperature and volume to continue the biochemical changes initiated during the malting process. The mashing process is conducted over a period of time at various temperatures in order to activate the endogenous malt enzymes responsible for the

degradation of proteins and carbohydrates. By far the most important change brought about in mashing is the conversion of starch molecules into fermentable sugars. The principal enzymes responsible for starch conversion in a traditional mashing process are alpha- and beta-amylases. Alpha-amylase very rapidly reduces insoluble and soluble starch by splitting starch molecules into many shorter chains that can be attacked by beta-amylase. The disaccharide produced is maltose.

When brewing from grists low in enzymes such as high adjunct grists, mashing may be performed in the presence of added enzyme compositions comprising the enzymes necessary for the hydrolysis of the grist starch.

After mashing, it is necessary to separate the liquid extract (the wort) from the solids (spent grains i.e. the insoluble grain and husk material forming part of grist). Wort separation is important because the solids contain large amounts of non-starch polysaccharides, protein, poorly modified starch, fatty material, silicates, and polyphenols (tannins). Important non-starch polysaccharides present in cereal grains are beta-glucan and arabinoxylan. The objectives of wort separation, *inter alia*, include the following:

- to obtain good extract recovery,
- to obtain good filterability, and
- to produce clear wort.

Extraction recovery and filterability are important for the economy in the brewing process, while the wort clarity is a must in order to produce a beer which does not develop haze. Extraction recovery, filterability and wort clarity is greatly affected by the standard of the grist, e.g. the barley malt and the types of adjunct, as well as the applied mashing procedure.

Following the separation of the wort from the spent grains the wort may be fermented with brewers yeast to produce a beer.

A typical wort consists of a mixture of starch derived carbohydrates which are classified as fermentable or non-fermentable according to whether they can be converted into ethanol by brewer's yeast. In traditional mashing the fermentable carbohydrates are formed by hydrolysis of grain starch by malt alpha- and beta-amylases.

Starch is a glucose polymer in which the glucose residues are linked by either alpha-1,4 bonds or alpha-1,6 bonds. During the mashing cycle the starch is first solubilized and then a portion of the starch molecules are hydrolyzed into non-fermentable dextrins and to low molecular weight sugars, such as glucose, maltose and maltotriose, which brewers yeast can ferment into ethanol. The non-fermentable or limit dextrin fraction consists of all sugars with a higher degree of polymerisation (DP) than matotriose.

As indicated above the hydrolysis of the grist starch in traditional mashing is catalysed by two enzymes endogenous to malted barley. One, the alpha-amylase, randomly cleaves alpha-1,4 bonds in the interior of the raw, largely insoluble starch molecule fragmenting them into large but soluble polysaccharides termed dextrins. The second, beta-

amylase, is an exo-amylase which sequentially cleaves alpha-1,4 bonds from the non reducing end of the these dextrins producing mainly maltose.

Both alpha- and beta-amylase are unable to hydrolyse the alpha-1,6 bonds which forms the branching points of the starch chains in the starch molecule. As the enzymes are unable to debranch the starch molecule this results in the formation of the aforementioned limit dextrins.

The composition of the wort can vary depending on the starting material, mash cycles and other variables. The carbohydrate composition of a typical wort consists of 65-80% fermentable sugars, and 20-35% non-fermentable limit dextrins. During fermentation the fermentable fraction is converted into ethanol to a final concentration of 3 to 6 % w/w. The limit dextrins are not converted into ethanol and remains in the final beer adding to the carbohydrate content of the beverage.

In the production of "low carb" or super attenuated beers, an attempt is made to obtain a higher proportion of alcohol and a lower amount of residual dextrin. These beers are formulated using exogenous enzymes compositions comprising enzyme activities capable of debranching the limit dextrins. Glucoamylase, an enzyme often used in brewing compositions have some action towards alpha-1,6 bonds, and may be applied to reduce the content of limit dextrins. However, as glucoamylases are much more efficient in hydrolyzing the alpha-1,4 bonds and have difficulties in hydrolyzing the alpha-1,6 bonds, glucoamylases must be used in very high concentrations. Furthermore, the sugar profile obtained using high glucoamylase dosage is high in glucose and low in maltose, which is not considered optimal for the yeast.

The inventors of the present invention have found that by using an isoamylase a wort having a reduced amount of non-fermentable limit dextrins can be produced without the negative characteristics of the sugar profile obtained using high glucoamylase dosage.

Definitions

Throughout this disclosure, various terms that are generally understood by those of ordinary skill in the arts are used. Several terms are used with specific meaning, however, and are meant as defined by the following.

As used herein the term "**grist**" is understood as the starch or sugar containing material that's the basis for beer production, e.g. the barley malt and the adjunct.

The term "**malt**" is understood as any malted cereal grain, in particular barley.

The term "**adjunct**" is understood as the part of the grist which is not barley malt. The adjunct may be any carbohydrate rich material.

The term "**mash**" is understood as a aqueous starch slurry, e.g. comprising crushed barley malt, crushed barley, and/or other adjunct or a combination hereof, steeped in water to make wort.

The term "**wort**" is understood as the unfermented liquor run-off following extracting

the grist during mashing.

The term "**spent grains**" is understood as the drained solids remaining when the grist has been extracted and the wort separated from the mash.

The term "**beer**" is here understood as fermented wort, e.g. an alcoholic beverage brewed from barley malt, optionally adjunct and hops.

The term "**extract recovery**" in the wort is defined as the sum of soluble substances extracted from the grist (malt and adjuncts) expressed in percentage based on dry matter.

The term "**a thermostable enzyme**" is understood as an enzyme that under the temperature regime and the incubation period applied in the processes of the present invention in the amounts added is capable of sufficient degradation of the substrate in question.

The term "**homology**" when used about polypeptide or DNA sequences and referred to in this disclosure is understood as the degree of homology between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

The term "**DP**" is the degree of polymerisation, herein used for average number of glucose units in polymers in a polysaccharide hydrolysate.

The numbering of **Glycoside Hydrolase Families** (GH) applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *"Genetics, Biochemistry and Ecology of Cellulose Degradation"*., K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23, and in Bourne, Y. & Henrissat, B. 2001; Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600. This classification system groups glucoside hydrolases based on similarities in primary structure. The members of a family furthermore show the same catalytic mechanism and have similarities in the overall three-dimensional structure, although a family may contain members with substantial variation in substrate specificity.

The naming of *Humicola insolens* endoglucanases follows the system of Karlsson, J. 2000. Fungal Cellulases, Study of hydrolytic properties of endoglucanases from *Trichoderma reesei* and *Humicola insolens*. Lund University.

The inventors of the present invention have shown that isoamylase may be used in

a process for production of a wort having a low content of non-fermentable limit dextrins, and thus useful in the production of a beer having a low content of residual carbohydrates.

Embodiments of the invention

In a preferred embodiment of the first aspect the alpha-amylase is an acid fungal alpha-amylase, e.g. an acid fungal alpha-amylase derived from *A. niger*. In another preferred embodiment of the first aspect is a maltogenic alpha-amylase. In yet another preferred embodiment the process of the invention is performed in the presence of an acid fungal alpha-amylase as well as a maltogenic alpha-amylase.

In a further preferred embodiment of the first aspect one additional enzyme is present, which enzyme is selected from the list comprising; pullulanase, endoglucanase, xylanase, arabinofuranosidase, ferulic acid esterase, and xylan acetyl esterase. In a particularly preferred embodiment of the first aspect the endoglucanase is a Glucoside Hydrolase Family 12, 7 or 5 endoglucanase.

The process of the invention may be applied in the mashing of any grist. According to the invention the grist may comprise any starch and/or sugar containing plant material derivable from any plant and plant part, including tubers, roots, stems, leaves and seeds. Preferably the grist comprises grain, such as grain from barley, wheat, rye, oat, corn, rice, milo, millet and sorghum, and more preferably, at least 10%, or more preferably at least 15%, even more preferably at least 25%, or most preferably at least 35%, such as at least 50%, at least 75%, at least 90% or even 100% (w/w) of the grist of the wort is derived from grain. Most preferably the grist comprises malted grain, such as barley malt. Preferably, at least 10%, or more preferably at least 15%, even more preferably at least 25%, or most preferably at least 35%, such as at least 50%, at least 75%, at least 90% or even 100% (w/w) of the grist of the wort is derived from malted grain.

Preferably the grist comprises adjunct, such as non-malted grain from barley, wheat, rye, oat, corn, rice, milo, millet and sorghum, and more preferably, at least 10%, or more preferably at least 15%, even more preferably at least 25%, or most preferably at least 35%, such as at least 50%, at least 75%, at least 90% or even 100% (w/w) of the grist of the wort is derived from non-malted grain or other adjunct.

Adjunct comprising readily fermentable carbohydrates such as sugars or syrups may be added to the malt mash before, during or after the mashing process of the invention but is preferably added after the mashing process. A part of the adjunct may be treated with a protease and/or a endoglucanase, and/or heat treated before being added to the mash of the invention.

Other enzymes may be applied, preferably an enzyme, which is selected from the list comprising; pullulanase, endoglucanase, xylanase, arabinofuranosidase, ferulic acid esterase, and xylan acetyl esterase.

During the mashing process, starch extracted from the grist is gradually hydrolyzed

into fermentable sugars and smaller dextrins. Preferably the mash is starch negative to iodine testing, before wort separation.

Fermentation of the wort may include pitching the wort with slurry comprising fresh yeast, i.e. yeast not previously used for the invention or the yeast may be recycled yeast. The yeast applied may be any yeast suitable for beer brewing, especially yeasts selected from *Saccharomyces* spp. such as *S. cerevisiae* and S. *uvarum*, including natural or artificially produced variants of these organisms. The methods for fermentation of wort for production of beer are well known to the person skilled in the arts.

The process of the invention may include adding silica hydrogel to the fermented wort to increase the colloidal stability of the beer. The processes may further include adding kieselguhr to the fermented wort and filtering to render the beer bright. The beer produced by fermenting the wort of the invention may be any type of reduced carbohydrate beer, e.g. lager, pilsner, bitter, export beer, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer.

In a preferred embodiment of the second aspect the enzyme composition comprises one additional enzyme, which enzyme is selected from the list consisting of; pullulanase, endoglucanase, xylanase, arabinofuranosidase, ferulic acid esterase, and xylan acetyl esterase.

Isoamylase

The isoamylase to be used in the present invention may be of any origin including mammalian, plant or animal origin it is presently preferred that the isoamylase is of microbial origin. In particular the isoamylase may be one derivable from a filamentous fungus or a yeast. Preferably the isoamylase enzyme preparation is derivable from *Flavobacterium* sp. such as *F. odoratum* or from *Pseudomonas sp.,* such as from *P.amyloderamosa* and more preferably from the strain *P.amyloderamosa* ATCC21262.

Isoamylase activity is preferably used in amounts of 1 to 1.000.000.000 IAU/kg DS, more preferably 10 to 100.000.000 IAU/kg DS, even more preferably 100 to 10.000.000 IAU/kg DS, and most preferably 50.000 to 5.000.000 IAU/kg DS.

Isoamylase may be added in the amount of 0.001 mg to 100000 mg EP/kg DS, preferably in the amount of 0.01 mg to 10000 mg EP/kg DS, more preferably in the amount of 0.1 mg to 1000 mg EP/kg DS, most preferably in the amount of 1 mg to 100 mg EP/kg DS.

Endoglucanase

While the endoglucanase to be used for the present invention may be of any origin including mammalian, plant or animal origin it is presently preferred that the endoglucanase is of microbial origin. In particular the endoglucanase may be one derivable from a filamentous fungus or a yeast.

More preferably, according to the process of the invention the endoglucanase is

derived from a filamentous fungus such as from *Aspergillus* sp. or *Humicola* sp. and preferably from *Aspergillus aculeatus* or *Humicola insolens.*

Preferred GH12 glucanases includes endoglucanases obtained from *Aspergillus* sp. such as from *Aspergillus kawachii* (SWISSPROT Q12679), or *Aspergillus niger* (SWISSPROT O74705), *Aspergillus oryzae* (SWISSPROT O13454), from *Erwinia* sp., such as from *Erwinia carotovora* (SWISSPROT P16630), and from *Thermotoga* sp., such as from *Thermotoga maritima* (SWISSPROT Q60032 or Q9S5X8). Also preferred are any sequence having at least 50%, at least 60%, at least 70%, at least 80%, or even at least 90% homology to any of the aforementioned GH12 glucanases sequences.

Preferred GH7 glucanases includes endoglucanases obtained from *Agaricus* sp., such as from *Agaricus bisporus* (SWISSPROT Q92400), from *Aspergillus* sp., such as from *Aspergillus niger* (SWISSPROT Q9UVS8), from *Fusarium* sp., such as from *Fusarium oxysporum* (SWISSPROT P46238), from *Neurospora* sp., such as from *Neurospora crassa* (SWISSPROT P38676), and from *Trichoderma* sp., such as from *Trichoderma longibrachiatum* (SWISSPROT Q12714). Also preferred are any sequence having at least 50%, at least 60%, at least 70%, at least 80%, or even at least 90% homology to any of the aforementioned GH7 glucanases sequences.

Preferred GH5 glucanases includes endoglucanases obtained from *Acidothermus* sp., such as from *Acidothermus cellulolyticus* (SWISSPROT P54583), from *Aspergillus* sp., such as from *Aspergillus niger* (SWISSPROT O74706), and from *Bacillus* sp., such as from *Bacillus polymyxa* (SWISSPROT P23548). Most preferred is a beta-glucanase derived from *Thermoascus aurantiacus*, such as from the strain IFO 9748 (BG025) and having the amino acid sequence submitted to NCBI Entrez Protein Database (accession no. GenPept AAL 16412.1) on 10-SEP-2001. Also preferred are any sequence having at least 50%, at least 60%, at least 70%, at least 80%, or even at least 90% homology to any of the aforementioned GH5 glucanases sequences.

Endoglucanase activity may be added in the amount of 0.001 mg to 100000 mg EP/kg DS, preferably in the amount of 0.01 mg to 10000 mg EP/kg DS, more preferably in the amount of 0.1 mg to 1000 mg EP/kg DS, most preferably in the amount of 1 mg to 100 mg EP/kg DS.

Xylanase

While the xylanase to be used for the present invention may be of any origin including mammalian, plant or animal origin it is presently preferred that the xylanase is of microbial origin. In particular the xylanase may be one derivable from a filamentous fungus or a yeast.

Xylanases have been found in a number of fungal species, in particular species of *Aspergillus*, such as *A. niger*, *A. awamori*, *A. aculeatus and A. oryzae*, *Trichoderma*, such as *T. reesei* or *T. harzianum, Penicillium*, such as *P. camenbertii, Fusarium*, such as *F. oxysporum*, *Humicola*, such as *H. insolens,* and *Thermomyces,* such as *T. lanuginosa.* Xylanases have

also been found in bacterial species, e.g. within the genus *Bacillus*, such as *B. pumilus*.

Preferably, according to the process of the invention the xylanase is derived from a filamentous fungus such as from *Aspergillus* sp., *Bacillus sp., Humicola* sp., *Myceliophotora* sp., *Poitrasia* sp. or *Rhizomucor* sp.

Preferably the xylanase to be used in the present invention is a Glycoside Hydrolase Family 10 (GH10) xylanase. Preferably, according to the process of the invention the xylanase is derived from *Aspergillus aculeatus, and preferably the* xylanase *is a* GH10 xylanase. Most preferably the xylanase is derived from *Aspergillus aculeatus* CBS 101.43, and most preferably the xylanase has comprising the partial amino acid sequence shown in SEQ ID NO:5 in WO9421785A1.

Also preferred are any sequence having at least 50%, at least 60%, at least 70%, at least 80%, or even at least 90% homology to any of the aforementioned xylanase sequence.

Xylanase activity may be added in the amount of 0.001 mg to 100000 mg EP/kg DS, preferably in the amount of 0.01 mg to 10000 mg EP/kg DS, more preferably in the amount of 0.1 mg to 1000 mg EP/kg DS, most preferably in the amount of 1 mg to 100 mg EP/kg DS.

Glucoamylases

A glucoamylase (E.C.3.2.1.3) to be used in the processes of the invention may be derived from a microorganism or a plant. Preferred is glucoamylases of fungal origin such as *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102). Also preferred are variants thereof, such as disclosed in WO92/00381 and WO00/04136; the *A. awamori* glucoamylase (WO84/02921), *A. oryzae* (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof. Preferred glucoamylases include the glucoamylases derived from *Aspergillus niger*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence set forth in WO00/04136 and SEQ ID NO: 13. Also preferred are the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence set forth in WO00/04136 SEQ ID NO:2.

Other preferred glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO99/28448), *Talaromyces leycettanus* (US patent no. Re.32,153), *Talaromyces duponti, Talaromyces thermophilus* (US patent no. 4,587,215), *Clostridium*, in particular *C. thermoamylolyticum* (EP135,138), and *C. thermohydrosulfuricum* (WO86/01831).

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN EXTRA L and AMG™ E (from Novozymes A/S); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

Glucoamylase activity may be used in the amounts of 0.1 to 100000 AGU/kg DS,

preferably in the amounts of 1 to 10000 AGU/kg DS, more preferably in the amounts of 10 to 1000 AGU/kg DS, such as 100 to 500 AGU/kg DS.

Glucoamylase may be added in the amount of 0.001 mg to 100000 mg EP/kg DS, preferably in the amount of 0.01 mg to 10000 mg EP/kg DS, more preferably in the amount of 0.1 mg to 1000 mg EP/kg DS, most preferably in the amount of 1 mg to 100 mg EP/kg DS.

Alpha-amylase

The alpha-amylase to be used in the processes of the invention may be an acid fungal alpha-amylase, such as an acid fungal alpha-amylase obtained from a strain of *Aspergillus, preferably* a strain of *Aspergillus niger* or a strain of a strain of *Aspergillus oryzae.* More preferably the acid alpha-amylase is an acid alpha-amylase having at least at least at least 70% homology, such as at least 80% or even at least 90% homology to the acid fungal alpha-amylase having the amino acid in the sequence shown in SWISPROT No: P10529.

Preferred alpha-amylases for use in the present invention also comprise a bacterial alpha-amylases, such as an alpha-amylase derived from a strain of *B. licheniformis, B. amyloliquefaciens,* or *B. stearothermophilus.* Preferably the acid alpha-amylase used for the process of the invention is one of the acid alpha-amylase variants and hybrids described in WO96/23874, WO97/41213, and WO99/19467, such as the *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variant having the following mutations delta(181-182) + N193F (also denoted I181* + G182* + N193F).

Preferred commercial compositions comprising alpha-amylase include Mycolase from DSM (Gist Brochades), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X and SAN™ SUPER, SAN™ EXTRA L (Novozymes A/S) and Clarase L-40,000, DEX-LO™, Spezyme FRED, SPEZYME™ AA, and SPEZYME™ DELTA AA (Genencor Int.).

The alpha-amylase to be used in the processes of the invention may preferably be a maltogenic alpha-amylase (E.C. 3.2.1.133). Maltogenic alpha-amylases (glucan 1,4-alpha-maltohydrolase) are able to hydrolyse amylose and amylopectin to maltose in the alpha-configuration. Furthermore, a maltogenic alpha-amylase is able to hydrolyse maltotriose as well as cyclodextrins. Specifically contemplated maltogenic alpha-amylases may be derived from *Bacillus* sp., preferably from *Bacillus stearothermophilus*, most preferably from *Bacillus stearothermophilus* C599 such as the one described in EP120.693. This particular maltogenic alpha-amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628. A preferred maltogenic alpha-amylase has an amino acid sequence having at least 70% identity to amino acids 1-686 of SEQ ID NO:1 in US6162628, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99%. Most preferred variants of the maltogenic alpha-amylase comprise the variants disclosed in WO99/43794.

The sugar profiles obtained using a maltogenic alpha-amylase will be rich in maltose which will be beneficial for the yeast. Commercially available compositions comprising maltogenic alpha-amylase include NOVAMYL™ from Novozymes A/S.

Maltogenic alpha-amylases may be added in amounts of 0.01-40.0 MANU/g DS, preferably from 0.02-10 MANU/g DS, preferably 0.05-5.0 MANU/g DS. Maltogenic alpha-amylases may be added in amounts 0.001 to 100000 mg EP/kg DS, preferably 0.01 to 10000 mg EP/kg DS, more preferably 0.1 to 1000 mg EP/kg DS, and most preferably 1 to 100 mg EP/kg DS.

MATERIALS AND METHODS

Isoamylase activity

Isoamylase activity may be expressed in IAU (IsoAmylase Units) which is the amount of enzyme which causes an increase in absorbency of 0.01 at 610 nm under the following standard conditions:

A reaction mixture comprising 1 ml of a suitably diluted enzyme solution, 5 ml of 1% amylopectin (waxy-maize starch Snowflake™ 04201 – CPC) solution, and 1 ml of 0.5 M acetate buffer solution pH 3.5 is incubated at 40°C for 30 minutes.

0.5 ml of the reaction mixture is withdrawn and mixed with 0.5 ml of 0.01 M Iodine solution and 15 ml of 0.02 N H_2SO_4 solution. After 15 minutes the absorbency at a wave length of 610 is determined.

Xylanolytic activity

The xylanolytic activity can be expressed in FXU(S)-units, determined at pH 6.0 with remazol-xylan (4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R, Fluka) as substrate.

A xylanase sample is incubated with the remazol-xylan substrate. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant (as determined spectrophotometrically at 585 nm) is proportional to the xylanase activity, and the xylanase units are then determined relatively to an enzyme standard at standard reaction conditions, i.e. Substrate concentration 0.45% w/v, Enzyme concentration 0.04 – 0.14 FXU(S)/mL at 50.0 °C, pH 6.0, and in 30 minutes reaction time. Xylanase activity in FXU(S) is measured relative to a Novozymes FXU(S) enzyme standard comprising the monocomponent xylanase preparation Shearzyme from *Aspergillus aculeatus.*

A folder EB-SM-0397.02 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the

reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum solubile.

A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 FAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrins and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

$$\text{STARCH} + \text{IODINE} \xrightarrow[40^\circ,\ \text{pH}\ 2{,}5]{\text{ALPHA - AMYLASE}} \text{DEXTRINS} + \text{OLIGOSACCHARIDES}$$

$\lambda = 590\ \text{nm}$

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I2):	0.03 g/L
CaCl2:	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is

available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Glucoamylase activity (AGU)

The glucoamylase activity may be measured in AGU. One AGU is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A folder (AEL-SM-0131) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Endoglucanase activity

The cellulytic activity may be measured in fungal endoglucanase units (FBG), determined on a 0.5% beta-glucan substrate at 30°C, pH 5.0 and reaction time 30 min. Fungal endoglucanase reacts with beta-glucan releases glucose or reducing carbohydrate which is determined as reducing sugar according to the Somogyi-Nelson method.

1 fungal endoglucanase unit (FBG) is the amount of enzyme which according to the above outlined standard conditions, releases glucose or reducing carbohydrate with a reduction capacity equivalent to 1 micromol glucose per minute.

Pullulanase Activity (PUN)

Pullulanase activity may be determined relative to a pullulan substrate. Pullulan is a linear D-glucose polymer consisting essentially of maltotriosyl units joined by 1,6- alpha -links. Endopullulanases hydrolyze the 1,6-alpha-links at random, releasing maltotriose, 6^3- alpha -maltotriosyl-maltotriose, 6^3- alpha -(6^3- alpha -maltotriosyl-maltotriosyl)-maltotriose, etc. the number of links hydrolyzed is determined as reducing carbohydrate using a modified Somogyi-Nelson method.

One pullulanase unit (PUN) is the amount of enzyme which, under standard conditions (i.e. after 30 minutes reaction time at 40°C and pH 5.0; and with 0.2% pullulan as substrate) hydrolyzes pullulan, liberating reducing carbohydrate with a reducing power equivalent to 1 micromol glucose per minute.

A folder, AF 190/2-GB, describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Pullulanase may also be expressed in PUN(R) which is measured relative to a Novozymes A/S Promozyme standard.

A folder, EB-SM-0425.02/01, describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Enzymes used

A GH10 xylanase from *Aspergillus aculeatus* CBS 101.43 comprising the partial amino acid sequence shown in SEQ ID NO:5.in WO9421785A1.

An beta-glucanase derived from *Thermoascus aurantiacus* IFO 9748 (BG025) having the amino acid sequence submitted to NCBI Entrez Protein Database and having the accession no. GenPept AAL 16412.1.

An alpha-amylase derived from *B. amyloliquefaciens* (BAN 480 L).

An acid fungal alpha-amylase derived from *A.niger* having the amino acid sequence SWISPROT No: P10529.

An isoamylase enzyme preparation from *Pseudomonas amyloderamosa* ATCC21262.

A pullulanase having the amino acid sequence shown as SEQ ID NO:11 in US 5,736,375 or a pullulanase from *Bacillus acidopullulyticus* (Promozym 400 L, available from Novozymes)

A glucoamylase derived from A. niger (AMG 300 L, available from Novozymes).

A protease from *Thermoascus aurantiacus* (AP025) having the acid sequence shown in SEQ ID NO:2 in WO2003048353A1.

Methods

Mash preparation

Unless otherwise stated mashing was performed as follows. Except when noted the mash was prepared according to EBC: 4.5.1 using malt ground according to EBC: 1.1. Mashing trials were performed in 500 ml lidded vessels incubated in water bath with stirring and each containing a mash with 50 g grist and adjusted to a total weight of 300±0.2 g with water preheated to the initial incubation temperature + 1°C.

Mashing temperature profile

Unless otherwise stated mashing was carried out using an initial incubation temperature at 50°C for 30 minutes, followed by a 16 min temperature increase to 66°C and remaining here for 41 min. The profile is continued with a 10 min temperature increase to 75°C and remaining here for 10 min. All step wise temperature gradients are achieved by an increase

of 1°C/min. The mash is cooled to 20°C during 15 min, which result in a total incubation period of 121 min.

Additional methods

Methods for analysis of raw products, wort, beer etc. can be found in *Analytica-EBC*, Analysis Committee of EBC, the European Brewing Convention (1998), Verlag Hans Carl Geranke-Fachverlag. For the present invention the methods applied for determination of the following parameters were as indicated below.

Extract recovery: EBC: 4.5.1 (Extract of Malt: Congress Mash, Extract in dry). The term extract recovery in the wort is defined as the sum of soluble substances (glucose, sucrose, maltose, maltotriose, dextrins, protein, gums, inorganic, other substances) extracted from the grist (malt and adjuncts) expressed in percentage based on dry matter. The remaining insoluble part is defined as spent grains.

$$a)\ E_1 = \frac{P(M+800)}{100-P}$$

$$b)\ E_2 = \frac{E_1 \cdot 100}{100-M}$$

where;

E_1 = the extract content of sample, in % (m/m)

E_2 = the extract content of dry grist, in % (m/m)

P = the extract content in wort, in % Plato

M = the moisture content of the grist, in % (m/m)

800 = the amount of destilled water added into the mash to 100 g of grist

Example 1

Standard malt was used. Mash preparation and mashing profile were as described above. The enzymes were dosed at zero min in the mashing profile. The fermentable sugars, glucose, maltose, matotriose and fructose were quantified using HPLC. The results are shown in table 1.

Compared to the high glucoamylase treatment the amount of fermentable sugars can be increased 4-5% by using a combination of pullulanase and isoamylase. A corresponding decrease is observed in the amount of non-fermentable limit dextrins.

Table 1. Result of mashing trials. Enzyme dosages are given per kg DS grist. To all treatments a background dosage of xylanase 10 mg EP and betaglucanase 10 mg EP were added. DP4, DP4 and DP4+ is calc. relative to the amount in the AMG 3500 AGU treatment.

Additional enzymes	+ AMG 3500 AGU	+ Pullulanase 450 PUN+ AMG 3500 AGU	+ Pullulanase 450 PUN + AMG 1000 AGU	+ iso-amylase + AMG 3500 AGU	+ iso-amylase + AMG 1000 AGU	+ Pullulanase 450 PUN + Iso-
Extract recovery %	82,5	82,6	82,6	84,9	84,6	84,8
Glucose g/L:	23,99	25,33	16,92	26,58	16,59	17,22
Maltose g/L:	66,76	69,29	71,44	68,52	68,68	73,01
Maltotriose g/L:	9,51	9,75	14,81	9,34	14,10	15,33
Fructose g/L:	4,83	4,90	4,82	4,90	4,43	4,90
Sum of sugars g/L:	105,10	109,27	107,99	109,34	103,81	110,46
DP4	1	0.85	0.87	0.85	0.88	0.81
DP4 and DP4+	1	0.89	0.93	0.89	0.94	0.88

CLAIMS

1. A process for production of a beer having a low content of carbohydrates which comprises;

   a. preparing a mash in the presence of enzyme activities,

   b. filtering the mash to obtain a wort, and,

   c. fermenting said wort to obtain a beer

   wherein the enzyme activities comprise; an alpha-amylase, a glucoamylase and an isoamylase.

2. The process of the preceding claim wherein the alpha-amylase is an acid fungal alpha-amylase, e.g. an acid fungal alpha-amylase derived from *A. niger*.

3. The process of the preceding claims wherein the alpha-amylase is a maltogenic alpha-amylase.

4. The process of the preceding claims wherein at least one additional enzyme is present, which enzyme is selected from the list comprising; pullulanase, endoglucanase, xylanase, arabinofuranosidase, ferulic acid esterase, and xylan acetyl esterase.

5. The process of the preceding claims wherein the endoglucanase is a Glucoside Hydrolase Family 12, 7 or 5 endoglucanase.

6. An enzyme composition comprising an alpha-amylase, a glucoamylase and an isoamylase.

7. The enzyme composition of the preceding claim further comprising at least one additional enzyme, which enzyme is selected from the list comprising; pullulanase, endoglucanase, xylanase, arabinofuranosidase, ferulic acid esterase, and xylan acetyl esterase.

8. A use of the enzyme composition of the preceding claims in a mashing process.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000366

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12C5/00 C12C12/02 C12N9/30 C12N9/34 C12N9/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA, BIOSIS, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/011591 A (DOERRICH KURT ; HANNEMANN WOLFGANG (DE); FESTERSEN RIKKE (DK); NOVOZYM) 5 February 2004 (2004-02-05) page 12, line 27 - page 14, line 17; claims 1-5	1-8
A	US 4 666 718 A (DUNCOMBE GEORGE R ET AL) 19 May 1987 (1987-05-19) cited in the application examples	1-8
X	US 5 665 585 A (NEVALAINEN HELENA ET AL) 9 September 1997 (1997-09-09) column 18, lines 39-64	6,7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 July 2005	27/07/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Koch, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000366

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 354 828 A (RHONE POULENC CHIMIE) 14 February 1990 (1990-02-14) page 2, line 60 - page 3, line 2	6,7
A	WO 02/074895 A (FELBY CLAUS ; PEDERSEN SVEN (DK); NOVOZYMES AS (DK); OLSEN HANS SEJR ( ) 26 September 2002 (2002-09-26) claims 1-8	1-8

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000366

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		EP 1373539 A2	02-01-2004